

## Life Cycles in the Methanogenic Archaeobacterium *Methanosarcina mazei*<sup>†</sup>

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*Methanosarcina mazei* S6 and LYC were used to study the structure and differentiation of the aggregating methanogens. Cultures harvested under various conditions are described at the ultrastructural level. Cells of strain S6 are enclosed by a layer 12 nm thick in contact with the plasma membrane. In sarcinal colonies, cells are held in close association by a fibrous matrix up to 60 nm thick. Colony maturation was examined in strain S6 over a period of 1 year. Changes occurred in the shape and staining of individual cells. Also, various inclusion bodies were observed that either persist throughout colony maturation or are only found at certain growth stages. Two types of cores that are composed of double membranes in *M. mazei* S6 are described. One has a 90-nm diameter and contains electron-dense granules similar to those found in the cytoplasm. The other core type does not contain granules, is more numerous, and is found in older cultures. Two life cycles are described for *M. mazei* based on electron microscope examinations. A complex life cycle involving the release of single cells is described with two variations for strains S6 and LYC. When released cells of strain S6 are placed in fresh medium they can repeat the cycle. In addition, a limited cycle is described for both strains of *M. mazei*. This limited cycle contains the only sarcinal morphotypes observed in *M. barkeri*. When *M. mazei* S6 remains in the limited cycle and does not disaggregate in stationary phase, several types of possible resting forms are found.

The sarcina-forming methanogens display a cellular arrangement that is unique, and several members exhibit complex life cycles that are rare among archaeobacteria. For example, in the life cycle of *Methanosarcina mazei*, which has been partially described by using light microscopy, sedentary colonies break up into single cells that can grow into new colonies in fresh medium (13).

In 1980, Mah reported the first successful isolation of a pure culture of *M. mazei* (13). The life cycle of this isolate, designated S6, was also determined by light microscopy, which revealed three morphotypes at different growth stages. When cultures are young, cells are found in small sarcinal aggregates (type I) which measure up to 20  $\mu\text{m}$  and which may form larger aggregations with each other. As the cultures mature and enter stationary phase, sarcinal aggregates go through a transitory stage to form coccal cysts (type II). These are up to 100  $\mu\text{m}$  in diameter and may form larger aggregates up to several millimeters. Mah reported that when the coccal cysts are physically disrupted, a cyst wall breaks, releasing individual cocci (type III) which are 1 to 3  $\mu\text{m}$  in diameter. Each of these can grow into sarcinal forms when placed in fresh medium.

Several other strains of *M. mazei* have been reported: MC<sub>3</sub> (34), whose life cycle is apparently identical to that of S6; Juelich (25), whose life cycle has not yet been described; and LYC (12). Strain LYC exhibits only two morphotypes and has a unique mechanism of disaggregation. A sarcinal colony forms when the cells are grown at pH 6.0, but when the pH is raised to 7.0 the colony disaggregates into single cells. Apparently an enzyme is activated that hydrolyzes the matrix holding the colony together. Unfortunately, the single cells cannot presently be induced to form aggregates, so a complete life cycle cannot be delineated.

Zhilina (37) reported on three *Methanosarcina* morphotypes that were grown in an enrichment culture. Morphotype I corresponds to a large sarcinal aggregate that possesses a central cavity with a single pore. Morphotype II is a smaller aggregation of irregular cells tightly enclosed by matrix material. Type III is a single cell, which was later reported by Zhilina and Zavarzin (38) to also occur in groups forming loose aggregates or in tightly packed cysts. However, these authors do not indicate any developmental changes that occur between the various forms. This biotype may be a strain of *M. mazei*, but that has not been confirmed.

Sowers et al. (29) recently reported on the isolation and ultrastructure of another species of *Methanosarcina*, *Methanosarcina acetivorans*, which grows in marine environments as irregular, single, motile cells. The cytoplasm contains electron-dense granules and is enclosed by a plasma membrane and a closely associated protein layer 10 nm thick. When cultures enter stationary phase, the single cells begin to aggregate into loose associations that are eventually enclosed by a thin proteinaceous cyst wall. A matrix is not present, and when fresh medium is added the aggregates break up into single cells once again.

The ability of a colony-forming bacterium to dissociate into single cells in response to unfavorable environmental changes provides a unique means for dispersal. When cells regain a favorable habitat, they form colonies which attach via sedimentation and entanglement and are less likely to wash away. Indeed, colony-forming methanogens are major inhabitants of fixed-bed reactors where the fluid components are periodically flushed from the system (22, 39).

*Methanosarcina* spp. are of prime importance in the final steps of biomass conversion to methane in mesophilic environments, where up to 70% of the methane formed is derived from the acetate-using methanogens (14). These two qualities, the complex life cycle and the importance in bioconversion, warrant an in-depth examination of this group. This

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paper characterizes the ultrastructure and development of these aggregating methanogens.

## MATERIALS AND METHODS

**Cultures and growth conditions.** *M. mazei* S6 and LYC were examined. Colony development was examined only in strain S6. Two different cultures of the same S6 strain were used. One exhibited a complex life cycle, whereas the other grew only in sarcinal colonies in a limited cycle and proved to be a variant of S6. The variant strain was obtained when cultures were repeatedly transferred in the laboratory. Strain S6 was grown at pH 6.8 in B1 medium (1), modified by the method of Mah (13), with acetate or methanol used as energy sources. Strain LYC was grown at pH 6.0 in a medium described by Liu et al. (12), with H<sub>2</sub>, CO<sub>2</sub> or methanol used as energy sources.

Cultures were usually grown in serum tubes (Bellco Glass, Inc.), containing 9 ml of medium and sealed with butyl rubber stoppers. Inoculations were performed by passing 1 ml of a 10- to 14-day-old culture through the stoppers with 20- or 25-gauge needles. All cultures were incubated at 35°C without shaking.

Sarcina development from single cells was examined in a series of tubes inoculated with cultures that had been filtered through a 3- $\mu$ m-pore filter to remove any remaining sarcinal forms. Tubes were incubated from 1 to 3 days and were then prepared for transmission electron microscopy.

Sarcinal colony maturation in variant strain S6 grown on acetate was monitored by inoculating a series of tubes through a 5- $\mu$ m-pore filter to grow the smallest possible sarcinal forms. No growth occurred when filters with pores smaller than 5  $\mu$ m were used. (Single cells are not released by this variant.) Cells were collected at 5, 10, 12, 15, 21, 30, 90, 105, and 360 days and prepared for electron microscopy.

Colony disaggregation was monitored with strain S6, which spontaneously breaks apart into single cells. When a 200-ml culture grown in a 600-ml serum bottle showed the first signs of turbidity, the colonies were examined by using light microscopy, and the culture was prepared for electron microscopy.

Colony disaggregation in strain LYC was monitored by growing the cultures at pH 6.0 for 5 to 7 days and then raising the pH to 7.0 by adding Na<sub>2</sub>HCO<sub>3</sub> (10% in reduced medium). Cultures were collected at 0, 12, 24, 36, and 48 h and prepared for electron microscopy.

**Preparation for electron microscopy.** Samples were fixed, embedded, and sectioned as previously described (23). Micrographs were taken on a Hitachi HU-11E or a JEOL JEM 100-CX transmission electron microscope operated at 75 and 60 kV, respectively. For stereo micrographs, semithick sections (gold) were observed at a tilt angle of  $-5^\circ$  and  $+5^\circ$  with a goniometer stage (19). The accelerating voltage was increased to 100 kV.

**Freeze-fracture.** Cells were collected on a 2.3-mm-diameter, 400-mesh gold grid, sandwiched between two 3-mm gold disks that were immediately frozen in a propane jet freezing apparatus (16). The frozen disks were stored in liquid nitrogen until they were separated in a Balzers BA-360M freeze-fracture machine. The fracture faces were angle shadowed with platinum-carbon and replicated with carbon by using electron guns. Replicas were cleaned in 50% aqueous chromic acid for 2 h, rinsed, then cleaned with sodium hypochlorite (50% household bleach) overnight before being collected on carbon- and Formvar-coated copper grids for viewing with transmission electron microscopy.

## RESULTS

**Morphology of the complex life cycle. (i) Cocci.** Thin sections showed irregular and regular cells up to 1.2  $\mu$ m (Fig. 1) that differed in their degree of cytoplasmic staining. The nuclear region was evenly dispersed and was not readily apparent. Electron-dense granules present in the cytoplasm ranged from 40 to 100 nm. These were spheroid with very rough margins and were characteristically surrounded by an electron-transparent halo. In single cells, the granules were not evenly distributed but tended to group into clusters at the periphery of the cytoplasm. Larger inclusion bodies similar to polyphosphates were common. The cytoplasm was bound by a unit membrane that was not very prominent and an additional, closely associated layer 12 nm thick (Fig. 2) that might contain protein (31). Frequently material from this layer appeared to slough off the outer surface in strips that sometimes had a membranous appearance, and occasionally cells were seen that had lost most of this layer (not shown).

**(ii) Division patterns.** A matrix material coated the outside of the cell surface and was probably responsible for cell-cell adhesion during colony formation. Matrix development began as a fibrous layer close to the cell surface. During cell division and subsequent development into sarcinae, the matrix became thicker, denser, and more regular, somewhat resembling a gram-positive wall. As sarcinal colonies matured, however, the matrix became very irregular, as described below.

The first division was by symmetrical binary fission with the two daughter cells remaining in contact, presumably because of the tight association of the cross matrix. In thin sections, single cells initiated division, with the plasma membrane and matrix invaginating as an annulus across the equator of the cell (Fig. 3). After the septum had bisected the cell, the cross matrix thickened from the periphery inward until it was as thick as the outer matrix. The second divisions that give rise to four daughter cells were initiated in the same plane but took place perpendicular to the first cross septum (not shown). In thin sections of 3-day cultures sarcinae showed a fairly regular shape, but the division septa were askew (Fig. 4). Unlike the first division where the completed septum was almost in the same plane, the second divisions were completed in an asymmetrical manner, and two perpendicular septa were rarely seen.

Overall, the sarcinae were approximately 3.5  $\mu$ m, and the matrix was more condensed. Cytoplasmic granules were more numerous and tended to group towards the center of the cells. Further growth resulted in irregular masses at 4 days (not shown).

**(iii) Sarcinal colonies.** In thin sections sarcinal colonies (seen in their limited cycle) were composed of a heterogeneously staining population of cells that were more irregular than the earlier sarcinal forms. The matrix was approximately 40 nm thick and had a fibrous appearance. The granules were either dispersed throughout the cytoplasm or were concentrated at the cell center. Clear areas were found where inclusion bodies had been removed during processing. These were up to 0.8  $\mu$ m and probably contained polyphosphate (25).

A large number of curved tubules 14 nm thick were trapped between the protein layer and the matrix (Fig. 5 and 6). These were sometimes in close association with the cell surface. When there is a high concentration of these tubules they can intertwine with the fibers of the matrix. In cross section (Fig. 5) they are circular and approximately 15 nm in diameter. Stereo pairs of micrographs show the tubular

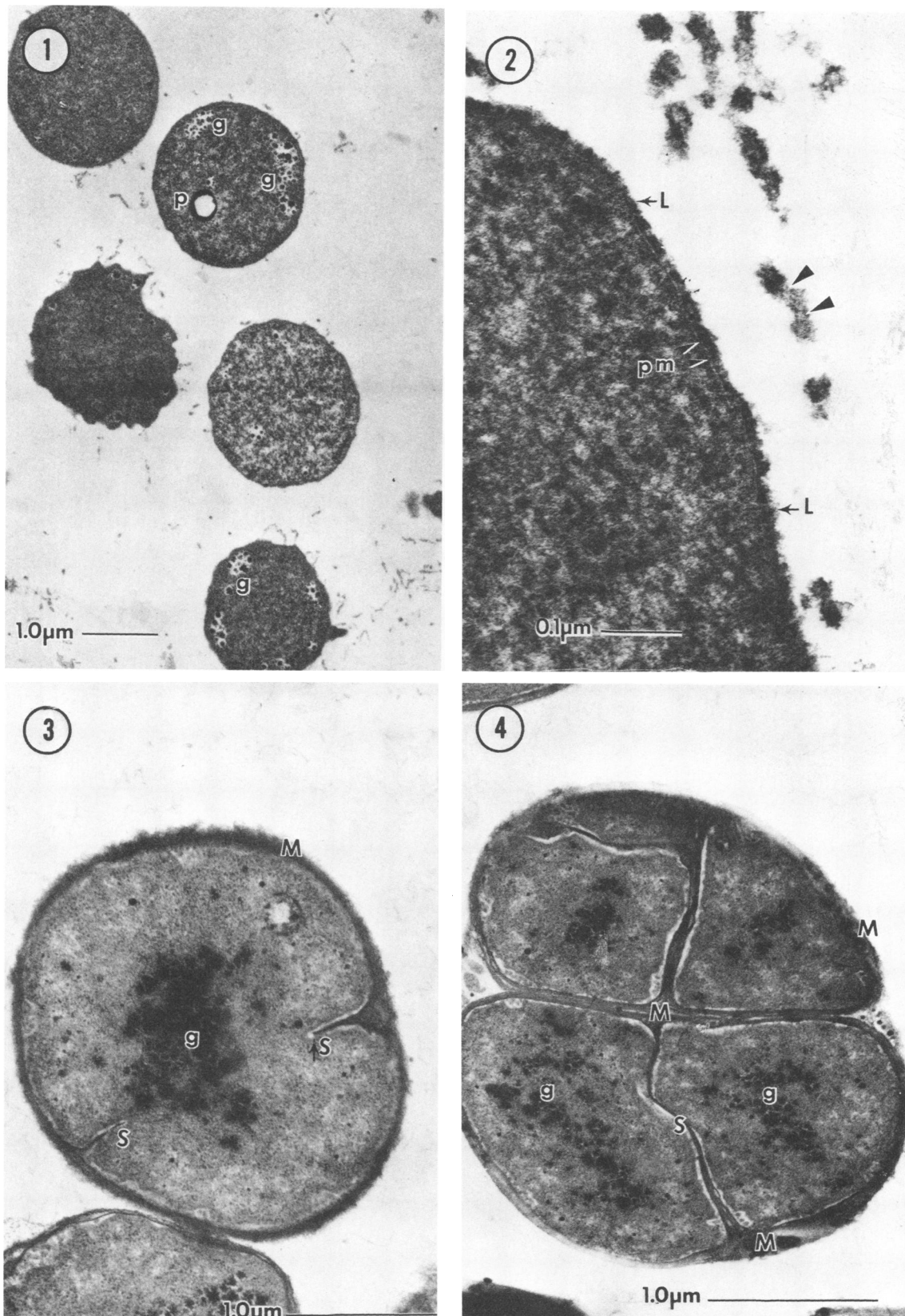


FIG. 1-4. Transmission electron micrographs of single and dividing cells of *M. mazei* S6. 1. Single cells containing cytoplasmic granules (g) and polyphosphate inclusion bodies (p). 2. Single cells showing a smooth surface with a layer (L) in close association with the plasma membrane (pm). Material with a membranous appearance is shedding from the cell surface (arrowheads). 3. A single cell initiating septum formation (S) which incorporates an outer matrix layer (M). 4. A sarcina showing the involvement of the matrix (M) in the cross septa (S). Numerous cytoplasmic granules (g) are present.

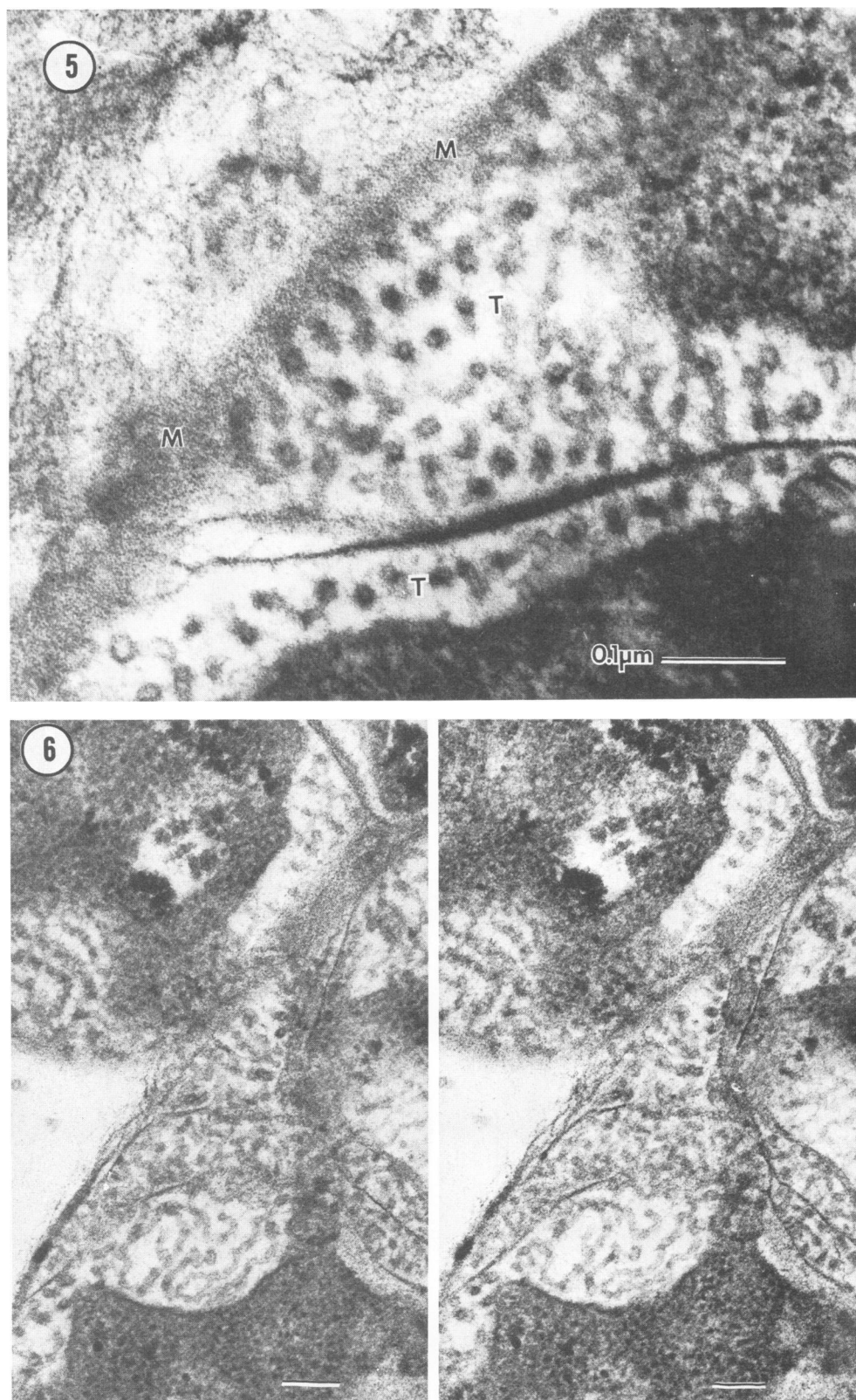


FIG. 5 and 6. Sections of *M. mazei* S6 showing the cell surface and the close association of membrane tubules. 5. High-magnification micrograph of a group of tubules (T) cut in cross section showing a hollow-tubule structure. These are located between the plasma membrane and the matrix material (M). 6. Stereo pair of micrographs clearly demonstrating the tubular structure of the membranous material. Bar = 0.1  $\mu\text{m}$ .





FIG. 7. A freeze-fracture replica of the cell surface showing specialized regions containing stacks of laminated material.

structures with an irregular arrangement, mixing with the fibers of the matrix (Fig. 6). Such structures are usually distributed in clusters at areas on the cell surface; however, they can also be found in grazing sections as threads stacked roughly parallel to each other and approximately 20 nm apart (not shown).

In freeze-fracture replicas of colonies growing in the complex cycle, specialized laminated structures were associated with the plasma membrane (Fig. 7). These usually consisted of stacks of material in curved or convoluted forms in which the individual layers were approximately 12 nm thick. Replicas of colonies grown in the limited cycle did not contain the specialized layered structures observed in replicas of colonies in the complex cycle. These structures may correlate to groups of tubules observed at the cell surface in thin sections. After cells mature into sarcinal colonies during the complex life cycle, they possess most of the ultrastructural characteristics of colonies that are restricted to the limited cycle described below.

(iv) **Colony disaggregation.** As reported earlier (23), the matrix begins shedding from cells, and single cells start streaming from colonies. Colony disaggregation is usually completed 48 h after initiation. However, coccal colonies are sometimes not completely degraded, and in such instances cells are held together by the remaining fragments of matrix. Slight physical pressure can break these colonies apart. Single cells within these colonies still possess a remnant of the matrix.

**Morphology of the limited life cycle.** (i) *M. mazei* S6. A limited cycle occurs in wild-type *M. mazei* when colonies are

still actively growing before matrix degradation and colony disaggregation begins. When wild-type *M. mazei* was repeatedly transferred in the laboratory, it gradually lost the ability to undergo the complex life cycle. It failed to disaggregate into single cells, and the matrix remained intact. This variant strain can only disperse by fragmentation of the sarcinal colony. *M. barkeri* and the variant *M. mazei* S6 are limited to this cycle and do not break up into individual cells. If these colonies are disturbed sufficiently by methane gas evolution or by shaking they will fragment into smaller masses. These will again grow into larger masses when placed in fresh medium.

In thin sections of *M. mazei* S6, the cytoplasm of the cells of 5-day-old colonies grown on acetate stained rather evenly (Fig. 8). At this stage the colonies were found singly in the culture medium and were uniform with a diameter of 3 to 5  $\mu\text{m}$ . The individual cells themselves were 0.6 to 1.0  $\mu\text{m}$  in diameter and were surrounded by a 30- to 60-nm matrix. The electron-dense granules were scattered throughout the cytoplasm, and polyphosphatelike bodies were rarely seen. Membrane tubules between the cell surface and the matrix were not commonly observed in cultures restricted to the limited cycle.

Within 10 days, the colonies enlarged to 8 to 10  $\mu\text{m}$  in diameter and the cells to 1.5  $\mu\text{m}$  in diameter (Fig. 9). Cells of this age showed a variability of cytoplasmic staining correlated with three morphological types. These cell types are arbitrarily defined, and intermediate forms can be found. Type A has a dense, rather dark-staining cytoplasm, whereas that of type B appears less dense and stains lightly. Both of these types sometimes contain membrane cores (see below) which include electron-dense granules (Fig. 9). Another cell type, type C, appears highly degenerative and is usually found near the periphery of the colony. This type has numerous inclusions and membranous structures and does not show the cytoplasm-staining homogeneity seen in younger-looking cells.

At 12 days the three cell types within a colony were present in approximately equal numbers, but by day 15 the lighter-staining cell type B predominated. In cultures older than 21 days the degenerative cell type was most common (not shown). At 105 and 360 days (not shown), by which time the cell colonies all appeared the same, only a few single cells looked viable. The matrix between degenerative cells was poorly defined, and the granules and polyphosphate bodies persisted. Possible resting forms (see below) can be found in these older cultures which have thick specialized walls.

(ii) **Possible resting forms.** Cultures of the variant strain S6 older than 3 months were largely composed of dead cells. However, several types of possible resting forms were found (Fig. 10 and 11). In thin sections, islands of viable-looking cells could be found toward the center of the colonies (Fig. 10). These were usually composed of 10 to 15 cells each and were surrounded by a dense layer of matrix material 100 to 200 nm thick. Frequently individual cells could be found with a specialized uniform layer 40 nm thick that was seemingly derived from the matrix but appeared more dense (Fig. 11). Division septa were never observed in these cells. The single-cell resting forms were usually spherical, but they were sometimes rod shaped. They sometimes contained cytoplasmic granules or polyphosphate bodies.

(iii) **Core structures.** Two kinds of membrane cores were found in the cytoplasm of cells in sarcinal colonies. One kind was found in dark-staining cell types A and B (Fig. 12 and 13). They were 90 nm in diameter and appeared to have a

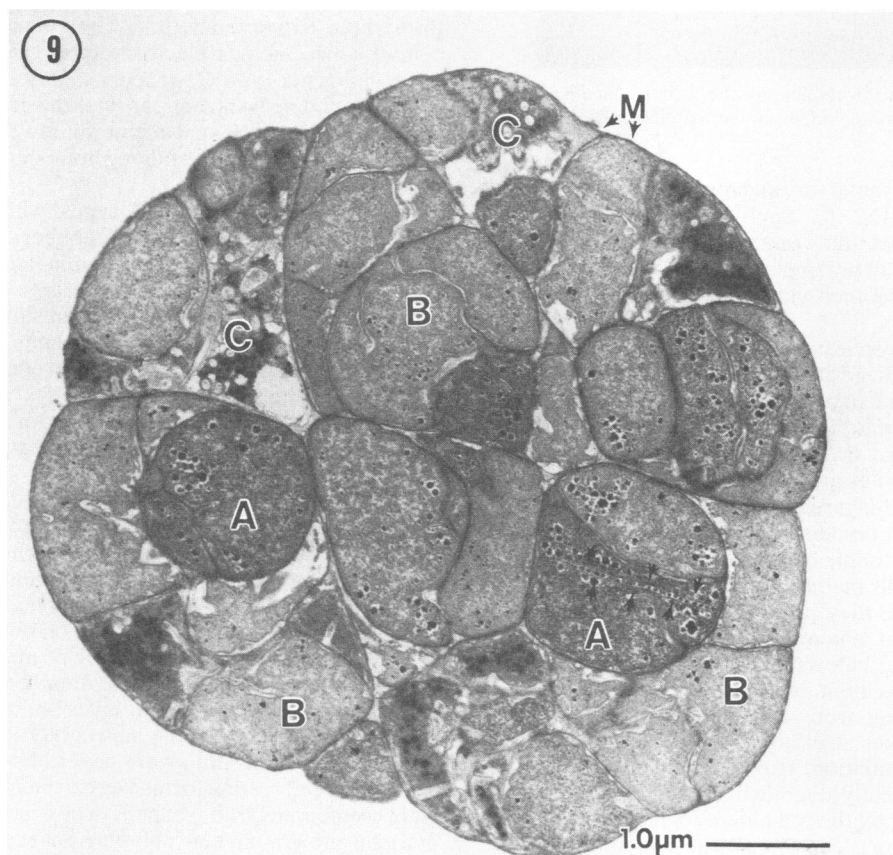
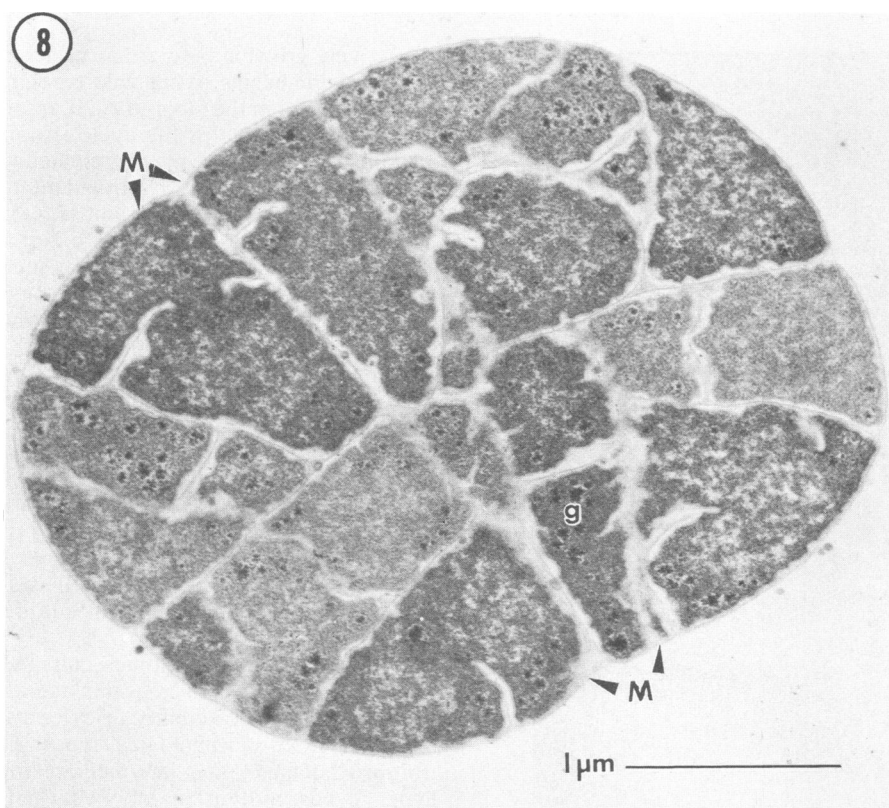


FIG. 8 and 9. Young colonies of the variant *M. maezi* S6 growing in the limited cycle. 8. Five-day-old colony with fairly uniform staining cells. These contain cytoplasmic granules (g) and are surrounded by a matrix material (M). 9. Ten-day-old colony showing a variability in the staining of cell cytoplasm. Cell type A stains rather dark, whereas cell type B stains lighter. Both types appear viable and may contain core structures (arrowheads). Cell type C is highly degenerative with numerous inclusion vesicles.

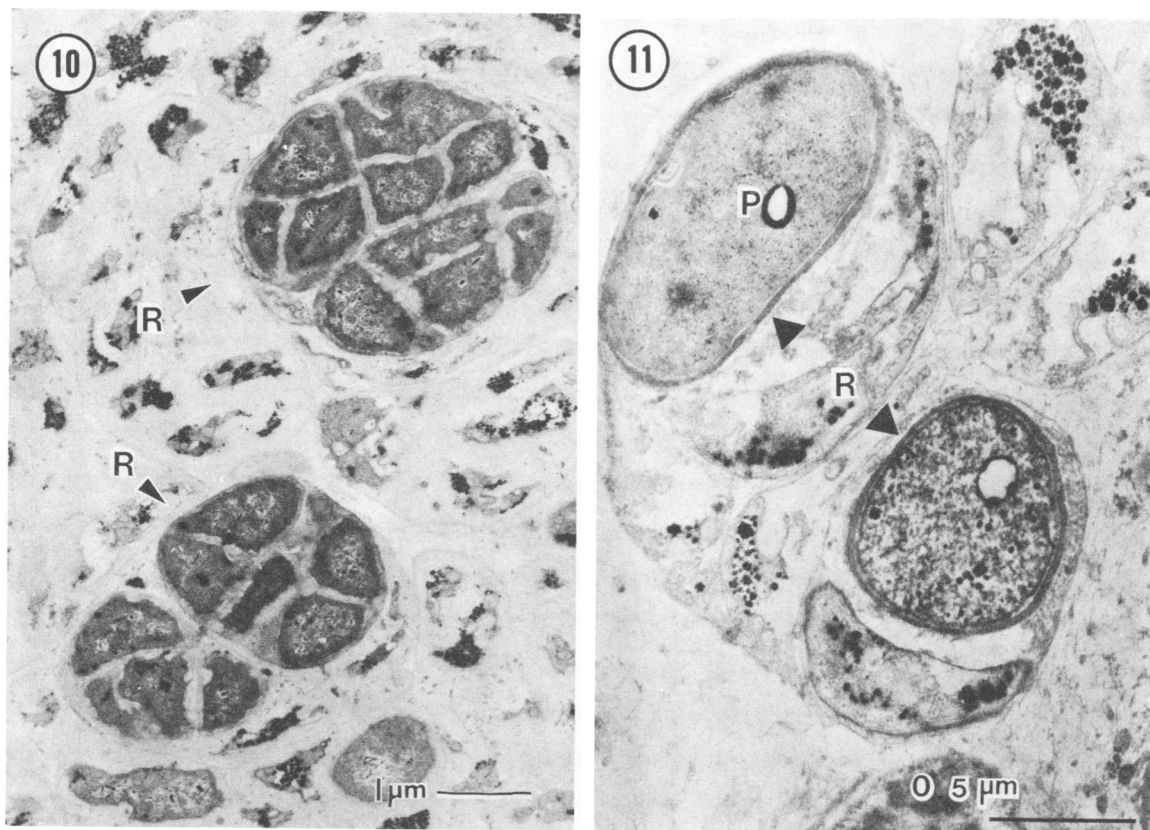


FIG. 10 and 11. Possible resting forms found in old cultures of *M. mazei* S6. 10. One type of resting form (R) composed of many cells enclosed by a thick specialized matrix. 11. Single-cell resting forms (R) are surrounded by a thick specialized layer and may contain polyphosphate inclusions (P).

double membrane surface 25 nm thick. A trilaminar unit structure was occasionally demonstrated (Fig. 13). There can be up to three cores of this kind per cell, and when more than one was present they were arranged parallel to each other (not shown). Inside the cylindrical cores were electron-dense granules arranged in single file. These granules were of the same appearance as those found in the cytoplasm but were much more uniform in size (40 nm in diameter). These cores were observed in less than 1% of the cells in thin sections but almost traversed the cytoplasm when they occurred and were closely associated with the plasma membrane. A connection with the plasma membrane was not observed, and the ends appeared to open into the cytoplasm.

The second kind of core was associated with the plasma membrane in cell type C (Fig. 14). These cores were 100 nm in diameter and were shorter than those seen in younger cells (see above). They were more numerous, however, and did not present an orderly arrangement. These cores did not possess electron-dense granules and appeared empty. One of the inner membranes was continuous with the plasma membrane (Fig. 14), and in grazing sections a regular helical pattern was observed. This regular pattern may indicate a regular structure in the layer closely associated with this inner membrane. The ends of the other membrane have not been resolved.

(iv) **Disaggregation of *M. mazei* LYC.** Strain LYC grown at pH 6.0 has a morphology and life cycle similar to those of *M. barkeri*. If cultures are maintained at pH 6.0 the larger colonies will fragment into smaller colonies when agitated.

When the pH of a culture is changed to pH 7.0, however, colonies begin shedding single cells (12).

When disaggregating colonies of strain LYC were observed in thin sections, cells were being released from the matrix at the colony surface, though only the outermost cell layers were affected (Fig. 15). The cells in the colony interior had a homogeneous staining cytoplasm which contained only a few electron-dense granules. The matrix was dark staining and was approximately 50 nm thick. It was fibrous and generally not in close association with the plasma membrane. The matrix had a loose appearance in the colony periphery and was approximately 150 nm thick. At the very outer edge the matrix thinned until the underlying cell broke through.

The sarcinal colonies of strain LYC sometimes completely dissociated over a period of 48 h. Usually, though, degradation stopped before the entire colony was broken up, and colonies, although reduced in size, were still seen at the bottom of culture tubes.

## DISCUSSION

Descriptions of developmental or life cycles in archaeobacteria are rare and are generally limited to the genus *Methanosarcina*. The complex life cycle involving the release of single cells may provide a mechanism for cell dispersal during unfavorable growth conditions, whereas the limited cycle facilitates colony division during growth in favorable conditions.

Results of this study show that single cells of *M. mazei* can

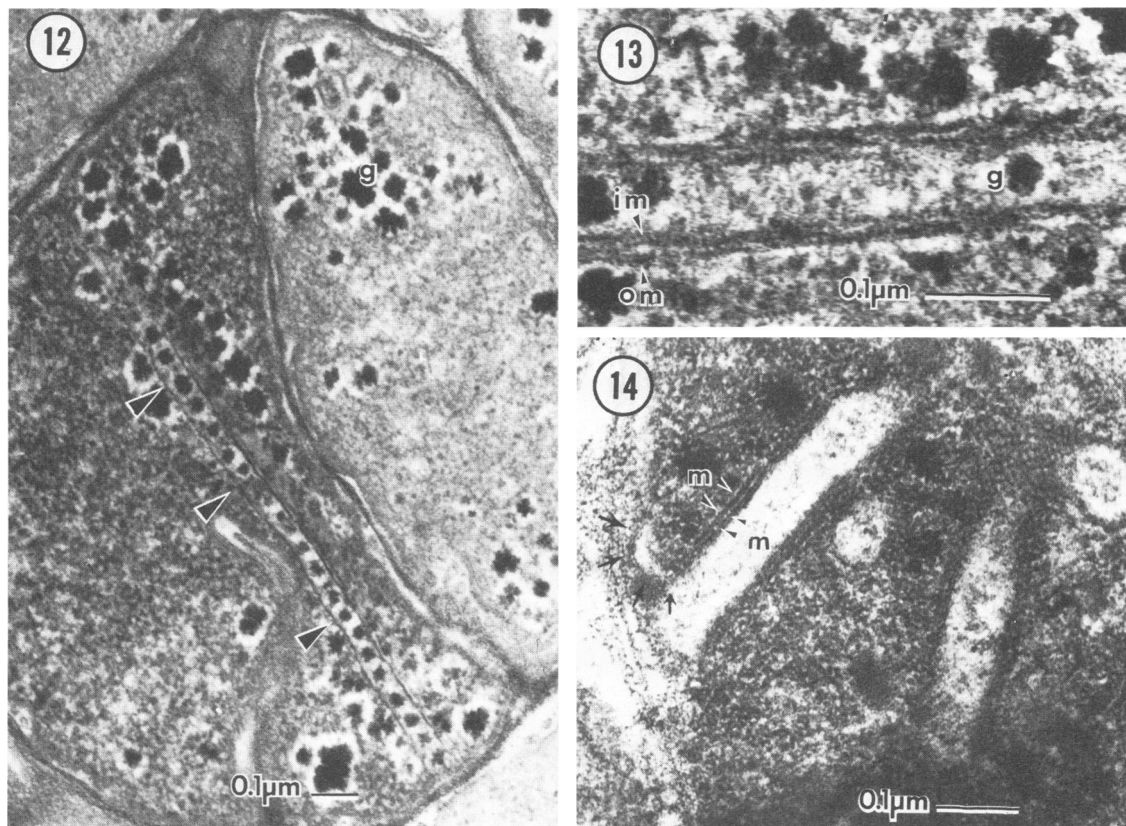


FIG. 12–14. Thin sections of membrane cores found in *M. mazei*. Fig. 12 and 13 show granule-containing cores found in cell types A and B (see the text). Fig. 14 shows plasma-membrane-associated cores found in cell type C. 12. This core nearly bisects the cell and contains a single file of cytoplasmic granules (g). 13. Granule-containing core at high magnification showing the inner (im) and outer (om) membranes of the core. 14. Cell containing several randomly arranged cores, one of which shows a connection with the plasma membrane (m and arrowheads).

be released via two different mechanisms (Fig. 16). When strain S6 is in stationary phase the matrix material begins to degrade, causing the colony to disaggregate into single cells (23). As the matrix degrades into a loose fibrillar network, the cells become less tightly associated within the colony. Alternatively, strain LYC grown at pH 6.0 produces a disaggregatase enzyme that is inactive until the pH is raised to 7.0 (12); then the matrix material at the periphery of the colony begins to be degraded. This results in a slow sequential release of single cells (Fig. 15). Introduction of the supernatant from strain LYC will also disrupt colonies of strain S6 in a similar fashion (12; unpublished data).

Although Mah reported that a cyst wall ruptures when single cells of *M. mazei* S6 are released, no structure was observed at the ultrastructural level enclosing the colonies. However, in examining thin sections of digester samples, groups of cells that are similar to *M. mazei* have been observed with a definite enclosing layer (unpublished data). Thus, the presence of a cyst wall may depend upon growth conditions.

The ultrastructure of single cells of *M. mazei* has not yet been reported, with the possible exception of *Methanosarcina* biotype 3, described by Zhilina and Zavarzin (38). These authors describe single cells approximately 1.0  $\mu\text{m}$  in diameter, enclosed by microcapsules of various thicknesses, which probably correspond to remaining matrix material. A cell wall near the protoplasm is described but is difficult to discern. This may be identical with the S6 protein layer

provisionally reported above, which is just outside the plasma membrane (Fig. 1 and 2).

This layer in *M. mazei* does not stain with silver methenamine (23), indicating absence of the reducing sugars that are present in the matrix. In thin sections of strain S6 this layer appears to shed from the surface as strands, and a few cells are nearly devoid of the layer. A trilaminar structure is observed in some of the material shedding from the surface (Fig. 2). This suggests that tubules may be shedding along with the surface layer. In micrographs of *M. acetivorans*, material also appears to be shedding from the surface layer (30). This shedding may be restricted among the archaeobacteria to cells of the methanosarcinae, since it is not evident in reports on other marine methanogens (4, 6–7, 8, 11, 20, 21, 24, 30).

In thin sections the first and second divisions are somewhat symmetrical, giving rise to tetrads (Fig. 4). These have a spherical surface, caused by the packing of matrix material. Zhilina and Zavarzin (38) describe a similar cellular arrangement for their biotype 3, which they call “pseudo-coccus,” because it appears as one large cell in the light microscope. In strain S6 the first two division planes have a symmetry similar to that of the divisions observed in the eubacterial sarcinae (5, 28, 33). However, the similarity is superficial since the matrix has a much more fibrous nature than a gram-positive wall does, and since a protein layer separates the matrix from the plasma membrane. The subsequent divisions are asymmetrical, thus preventing the



formation of regular packages of cells as seen in the eubacterial sarcinae.

The morphologies of sarcinal colonies in thin sections of strain S6 differed depending on which cycle the cultures were undergoing when harvested. Sarcinal colonies that developed from single cells during the complex cycle had a large number of tubules in localized areas between the cell surface and the matrix (Fig. 5 and 6). These may have shed from the surface layer and plasma membrane. Similar specialized structures associated with the plasma membrane have been called plasmalemmasomes in fungi (9, 15) and mesosomes in bacteria (2, 35). Both structures are believed to have specialized functions and may be involved with membrane storage. However, these structures are found in pockets of cytoplasm under the plasma membrane, whereas the structures described above project from the cell surface.

In freeze-fracture replicas, specialized structures closely associated with the plasma membrane sometimes contained neatly packaged material in strands approximately the same thickness as that of *Methanosarcina* tubules (Fig. 7). They may indeed be a close packing of the tubules. These specialized structures appear similar to the fractures of plasmalemmasomes observed in fungi by Marchant and Moore (15).

The number of tubules increased as the colonies aged, indicating that synthesis continues throughout the life cycle. Sarcinal colonies developed from variant strain S6 that exhibited only the limited life cycle showed small amounts of this material, which remained at a relatively low level during colony growth (Fig. 8 to 11). Since single cells are not



FIG. 15. Disaggregation in *M. mazei* LYC. The matrix in this strain is only degraded at the colony surface.

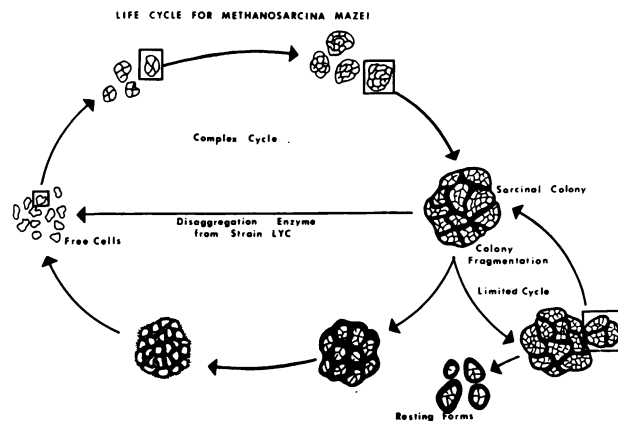


FIG. 16. Life cycle of *M. mazei*. Colonies develop from single cells through division and deposition of matrix material. Colonies can then multiply via fragmentation in a limited cycle and may become restricted to this cycle. In strain S6, single cells are released when the matrix degrades throughout the colony. In strain LYC, the matrix is degraded only at the colony surface. Resting forms are observed in older colonies of the variant strain S6.

released by these colonies, any benefits that these tubules have for the cell may be diminished by the persistence of the matrix, and the cells may be synthesizing less of this material.

The granule-containing cores observed in sarcinal colonies (Fig. 12 and 13) do not resemble any of the common bacterial inclusions previously described (27, 35). These cores consist of two membranes with a trilaminar unit structure. The cores nearly bisect the cytoplasm and are in close association with the plasma membrane. The ends are not clearly resolved but they apparently are not attached to the plasma membrane. The second type of core structure (Fig. 14) does not contain granules and is continuous with the plasma membrane and protein layer. Cells containing this type of core appear to be degenerative or dead.

Cores have been described in the streptococci by Coleman and Bleiweis (3). They can be common in that organism, depending on cellular growth conditions. However, these cores have up to three times the diameter and a thicker surface than do the sarcinal cores described above. They do not contain granules but consist mostly of protein, and their function is unknown.

The function of sarcinal granule-containing cores is also unknown, but since the granules are smaller and more uniform in size than the cytoplasmic granules, they may be involved in the sequestering of storage material during granule formation. Although they were observed in pure cultures of *M. mazei* S6 in less than 1% of the cells in thin section, they are much more prevalent in mixed cultures. This sections of digester samples have been observed where nearly 75% of the methanosarcinal cells possess one or more granule-containing cores (unpublished data).

There is no ultrastructural evidence that the cores are sites for methane production. In exponential-phase cultures they usually enclose electron-dense granules, which are probably storage products and may not be involved with methane production. The granule-containing cores in *M. mazei* are rarely observed in cells grown on methanol or trimethylamines (unpublished data). Hence, it is more likely that they are involved in granule synthesis since the enclosed granules are smaller than the free cytoplasmic granules.

Also, in this respect they would not be required at all times during the life cycle.

Sarcinal colonies of *M. mazei* S6 that remain in stationary phase for longer than 3 months contain several morphotypes of viable-looking cells that appear to be resting forms (Fig. 10 and 11). These are surrounded by matrixlike material that is thicker than that observed in younger cultures. While investigating cell death in *Methanosarcina* strains exposed to air, Zhilina (36) found specialized cystlike forms under light microscopy. These were larger than vegetative cells and appeared more dense. Zhilina and Zavarzin (38) later described a microcyst, at the ultrastructural level which is a single cell surrounded by a thick specialized wall. They did not speculate that this might be the same structure observed earlier in the light microscope.

The term resting form is used here to describe forms that meet six conditions. (i) Such forms do not resemble the endospores described for *Methanobacterium* MOH (31) or typical eubacterial spores. (ii) Cells remain viable in cultures for over 6 months. (iii) Viable-looking cells are present in thin sections of 1-year-old cultures. (iv) Cells have a thicker covering than vegetative cells (probably Zhilina's microcysts). (v) Cells occur as several different morphotypes. (vi) Cultures are more tolerant to oxygen exposure than other methanogens are. This last observation was the focus of a study by Kiener and Leisinger (10) who examined the oxygen sensitivity of several methanogens. *M. barkeri* was found to withstand up to a 30-h exposure to air. Kiener and Leisinger believed this was because of the large sarcinal arrangements of cells that slowed oxygen diffusion to the center of the colony. The large sarcinal colonies probably do provide some protection from oxygen diffusion, but the resting forms may provide long-term survival during oxygen exposure or nutrient depletion. Parkin et al. (18) found that if digesters poisoned by toxic heavy metals were left undisturbed they would regenerate after a lag period of up to 6 months. This ability may result from the persistence of resting forms in digesters and the ability of *Methanosarcina* spp. to sequester heavy metals from the environment (29).

At the present time, a complex life cycle has been described (or implied by the presence of single cells) only for *M. mazei*, which currently includes strains S6 (12), MC3, (35), and LYC (13). The undesignated *Methanosarcina* strains MB (17) and biotype 3 (39) should also be included in this list. *M. acetivorans* undergoes a reverse life cycle, which has been described above. *M. barkeri* is apparently restricted to the limited cycle that can also be observed in *M. mazei*.

The transition in *M. mazei* S6 from colonial to single-cell forms is triggered by unknown factors. If inducing conditions are found (carbon/nitrogen ratio, pH, calcium concentration, etc.), then large numbers of single cells could be harvested for fractionation. Alternatively, the disaggregation enzyme produced by *M. mazei* LYC (12) holds promise by allowing one to directly manipulate the life cycles of several *Methanosarcina* strains.

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